Trafficking defects of the Southeast Asian ovalocytosis deletion mutant of anion exchanger 1 membrane proteins

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Human AE1 (anion exchanger 1) is a membrane glycoprotein found in erythrocytes and as a truncated form (kAE1) in the BLM (basolateral membrane) of α-intercalated cells of the distal nephron, where they carry out electroneutral chloride/bicarbonate exchange. SAO (Southeast Asian ovalocytosis) is a dominant inherited haematological condition arising from deletion of Ala^{400}–Ala^{408} in AE1, resulting in a misfolded and transport-inactive protein present in the ovalocyte membrane. Heterozygotes with SAO are able to acidify their urine, without symptoms of dRTA (distal renal tubular acidosis) that can be associated with mutations in kAE1. We examined the effect of the SAO deletion on stability and trafficking of AE1 and kAE1 in transfected HEK-293 (human embryonic kidney) cells and kAE1 in MDCK (Madin–Darby canine kidney) epithelial cells. In HEK-293 cells, expression levels and stabilities of SAO proteins were significantly reduced, and no mutant protein was detected at the cell surface. The intracellular retention of AE1 SAO in transfected HEK-293 cells suggests that erythroid-specific factors lacking in HEK-293 cells may be required for cell-surface expression. Although misfolded, SAO proteins could form heterodimers with the normal proteins, as well as homodimers. In MDCK cells, kAE1 was localized to the cell surface or the BLM after polarization, while kAE1 SAO was retained intracellularly. When kAE1 SAO was co-expressed with kAE1 in MDCK cells, kAE1 SAO was largely retained intracellularly; however, it also co-localized with kAE1 at the cell surface. We propose that, in the kidney of heterozygous SAO patients, dimers of kAE1 and heterodimers of kAE1 SAO and kAE1 traffic to the BLM of α-intercalated cells, while homodimers of kAE1 SAO are retained in the endoplasmic reticulum and are rapidly degraded. This results in sufficient cell-surface expression of kAE1 to maintain adequate bicarbonate reabsorption and proton secretion without dRTA.

Key words: anion exchanger 1 (AE1), distal renal tubular acidosis (dRTA), kidney, membrane protein, Southeast Asian ovalocytosis (SAO).

INTRODUCTION

The SLC4 gene family encodes membrane proteins that mediate bicarbonate transport in mammalian cells, and is important for pH homoeostasis and maintaining cell volume. Human AE1 (anion exchanger 1; SLC4A1, Band 3), one of the most-studied members of the SLC4 family, is a 911-amino-acid membrane glycoprotein (Figure 1) that is expressed in the plasma membrane of erythrocytes, existing as dimers and tetramers. In tissues, CO_2 produced by respiration diffuses into erythrocytes. Carbonic anhydrase II, which binds to the C-terminal tail of AE1 (reviewed in [1,2]), converts CO_2 into bicarbonate. Bicarbonate ions are transported out of the erythrocyte by AE1, in exchange for chloride in an electroneutral one-to-one fashion, increasing the CO_2-carrying capacity of the blood. In the lung, the process is reversed, resulting in the release of CO_2 from erythrocytes, which is then expired. A kidney isoform of AE1 (kAE1) is expressed in the distal nephron in the BLM (basolateral membrane) of α-intercalated cells, where it mediates the exchange of chloride and bicarbonate. It is shorter than AE1 in the cytosolic N-terminus because of the use of an alternative promoter, and begins with Met^{66} in the human protein (Figure 1). Together with protons that are pumped out to the lumen by an apical H^+-ATPase, bicarbonate ions are reabsorbed into the blood, and urine is acidified.

Hydropathy analysis and topological studies have suggested that AE1 has 12–14 TMs (transmembrane segments) [3] (Figure 1). Both the 43 kDa N-terminal domain and short C-terminal tail are cytosolic. The N-terminal domain contains binding sites for the erythrocyte cytoskeleton, haemoglobin and glycolytic enzymes [3]. The C-terminal 52 kDa membrane domain carries out anion transport [4]. A single N-glycan is attached to the fourth extracellular loop at Asn^{642}.

Mutations in AE1 and kAE1 have been implicated in various haematological and renal diseases. Defective or loss of interaction between mutant AE1 and the erythrocyte cytoskeleton results in HS (hereditary spherocytosis) [5], where osmotically fragile spherocytes are trapped and destroyed in the spleen. Mutations in kAE1 can lead to dominant or recessive dRTA (distal renal tubular acidosis) [6], characterized by a failure to acidify urine. Mutations that cause HS, however, do not usually cause dRTA and vice versa. Different biosynthetic constraints in erythrocyte precursors and kidney cells may explain why the same mutations that lead to intracellular retention and/or degradation of one AE1 isoform do not have any apparent effect on the other.

SAO (Southeast Asian ovalocytosis) is a haematological condition that results from a deletion in AE1, involving Ala^{400}–Ala^{408} [7,8] in the boundary between the N-terminal cytosolic domain and the first TM (Figure 1). First reported in 1965 [9], SAO is found in regions where malaria is endemic, possibly providing a selective advantage against cerebral malaria in children [10]. The condition is dominant; all individuals with SAO are heterozygous for the allele. Aside from erythrocytes that are ovalocytic and

Abbreviations used: AE1, anion exchanger 1 (erythroid isoform); BLM, basolateral membrane; $C_{12}E_8$, octa(ethylene glycol) dodecyl ether; CFTR, cystic fibrosis transmembrane conductance regulator; CNX, calnexin; DMEM, Dulbecco's modified Eagle's medium; dRTA, distal renal tubular acidosis; EndoH, endoglycosidase H; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPA, glycophorin A; HA, haemagglutinin; HEK-293, human embryonic kidney; HS, hereditary spherocytosis; kAE1, anion exchanger 1 (kidney isoform); MDCK, Madin–Darby canine kidney; NHS-SS-biotin, sulpho-succinimidyl 2-(biotin-amido)ethyl-1,3-dithiopropionate; PNGase F, peptide N-glycosidase F; SAO, Southeast Asian ovalocytosis; TM, transmembrane segment; VSVG, vesicular stomatitis virus glycoprotein.

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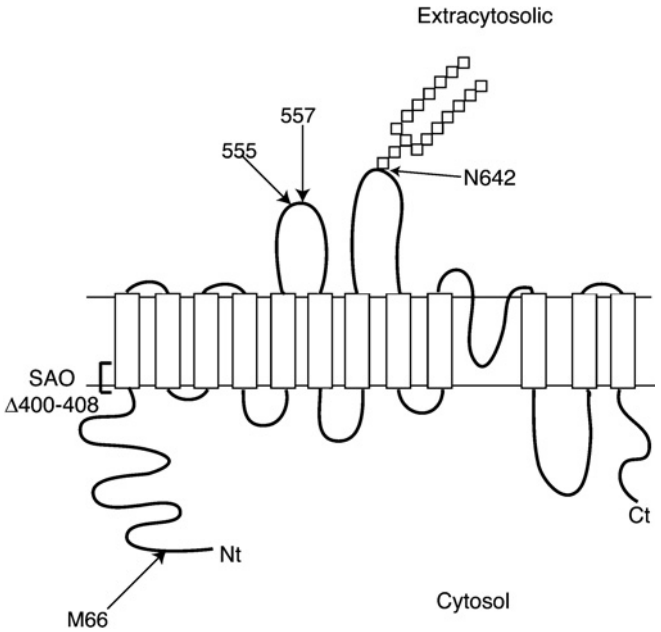

Figure 1 Folding model of human AE1

AE1 contains an N-terminal cytosolic domain and a membrane domain. KAE1 starts at Met^{66}. The membrane domain of human AE1 consists of 12 putative TMs. The endogenous N-glycosylation acceptor site (Asn^{642}) is indicated. Deletion of Ala^{400}–Ala^{408} results in SAO. The position of the novel N-glycosylation acceptor site (555) in the N555 constructs is shown. HA557 constructs contain a HA-tag after position 557. His_6-tagged constructs contain a hexahistidine tag after the last amino acid of the protein. Nt, N-terminus; Ct, C-terminus.

more rigid than normal [11], individuals with SAO are generally asymptomatic, with no anaemia or dRTA [12]. However, the homozygous state of SAO may be embryonic lethal [13]. SAO erythrocytes contain approximately equal amounts of AE1 and AE1 SAO [14]. Although AE1 SAO retains its normal secondary structure [14,15], the membrane domain is disordered [15], does not bind stilbene disulphonate inhibitors [14,15] and does not carry out anion transport [16]. The deletion affects the ability of the first TM to integrate into the membrane; however, it can still assume a transmembrane disposition [17]. AE1 SAO also lacks the polylactosaminyl N-glycan that is found on normal AE1, suggesting an effect of the deletion on oligosaccharide processing [14]. Heterodimers of AE1 and AE1 SAO in SAO erythrocytes were identified by cross-linking [18], and the interaction between normal and SAO subunits affects the structure of the normal subunit [19,20]. As SAO erythrocytes exhibit approximately half of the anion transport activity of normal erythrocytes [16], this suggests that the normal subunit is able to mediate transport, even when associated with an inactive SAO subunit. Whether AE1 SAO can form homodimers is not known.

Several studies have been carried out in *Xenopus* oocytes and transfected mammalian cell lines to understand the biosynthesis of AE1 SAO. AE1 SAO is able to traffic to the surface of oocytes [21,22] in the absence of normal AE1 or GPA (glycophorin A), but is inactive. However, co-injection of GPA cRNA enhances the trafficking of AE1 SAO to the surface of the oocyte [21], similar to the effect of GPA on normal AE1 [23]. Co-expressing AE1 and AE1 SAO in oocytes did not interfere with the trafficking of AE1 to the surface [22], although hetero-oligomerization was not shown in this study. AE1 SAO has also been stably expressed in K562 cells [24], a human erythroleukaemia cell line that expresses GPA [25]. The presence of Wr^b epitopes on K562 cells transfected with AE1 SAO demonstrates that AE1 SAO is able to interact with GPA. In addition, no difference was observed in the cell-surface expression between AE1 and AE1 SAO in transfected K562 cells as determined by binding of monoclonal anti-AE1 antibodies to intact cells.

Given the disordered state of the AE1 SAO membrane domain, we asked whether the inherent stability of AE1 SAO is different from that of AE1 in mammalian cells. If so, how does the mutant protein escape the quality-control system of the cell and traffic to the surface in erythrocyte precursors? One possibility is that in nature, SAO is always a heterozygous condition, and the presence of normal AE1 during biosynthesis may stabilize and rescue the cell-surface expression of AE1 SAO. Alternatively, erythroid-specific mechanism(s) may be in place to rescue the effect of misfolding on the biosynthesis of AE1 SAO. To answer these questions, erythroid and kidney forms of AE1 and AE1 SAO were expressed in HEK-293 (human embryonic kidney) cells and kAE1 SAO in MDCK (Madin–Darby canine kidney) cells. HEK-293 cells are a generic cell line lacking erythroid proteins such as GPA and were used to simulate heterozygous and homozygous conditions of SAO in the absence of erythroid-specific mechanisms. MDCK cells can form polarized monolayers and were used to simulate the environment in kidney cells to study kAE1 SAO biosynthesis, which has not been previously examined. We found that SAO proteins were less stable than the normal proteins, and did not traffic to the cell surface in the cell lines used in the present study. The presence of normal kAE1 could rescue cell-surface expression of kAE1 SAO in polarized MDCK cells. These results point to the possibility that erythroid-specific factors are involved in alleviating the biosynthesis defect of SAO protein in erythrocyte precursors. In the kidney of SAO patients, while kAE1 SAO is defective in trafficking to the BLM of α-intercalated cells in the kidney, its trafficking may be rescued by heterodimer formation with kAE1. kAE1 dimers that form would traffic normally to the BLM.

MATERIALS AND METHODS

Materials

The following is a list of materials used and their suppliers: pcDNA3 (Invitrogen); retroviral plasmids pVpack-GP, pVpack-VSVG (vesicular stomatitis virus glycoprotein) and pFBNeo-kAE1HA557, and QuikChange™ site-directed mutagenesis kit (Stratagene); FuGene 6 Transfection reagent (Roche Technologies); polybrene and geneticin G418 (Sigma); semi-permeable Transwell polycarbonate filters (Corning); DMEM (Dulbecco's modified Eagle's medium), DMEM/F-12, calf serum, penicillin, streptomycin and 10× Hanks balanced salt solution (Gibco BRL); EasyTag™ EXPRE^{35}S^{35}S protein labelling mix (PerkinElmer Life Sciences); MG262 (BIOMOL International); Protein G–Sepharose (Amersham Biosciences); EndoH (endoglycosidase H), PNGase F (peptide N-glycosidase F) and goat peroxidase-conjugated anti-rabbit IgG (New England Biolabs); $C_{12}E_8$ [octa-(ethylene glycol) dodecyl ether] (Nikko Chemical Co.); chemiluminescence kit (Boehringer Mannheim); ImmunoPure® immobilized streptavidin, EZ-Link™ NHS-SS-biotin [sulphosuccinimidyl 2-(biotin-amido)ethyl-1,3-dithiopropionate] and protein desalting spin columns (Pierce Biotechnology); Ni-NTA (Ni^{2+}-nitrilotriacetate)–agarose (Qiagen); mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Chemicon International); rabbit anti-CNX (calnexin) antibody (Stressgen Biotech); mouse anti-E-cadherin antibody (a gift from Dr Gergely L. Lukacs, University of Toronto, Toronto, Canada, and Dr W. J. Gallin, University of Alberta, Alberta, Canada); Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch

Laboratories); Alexa Fluor® 488-conjugated anti-mouse antibody (Molecular Probes); mouse anti-HA (haemagglutinin) antibody (Covance); rabbit anti-myc antibody (Santa Cruz Biotechnology); N70S CellMicroSieves of 70 μm pore size (BioDesign); and dropping bottle with 20–30 μm filter unit (Wheaton); mutagenic primers (ACGT Corp.).

Site-directed mutagenesis

The entire coding sequence for wild-type human AE1 was inserted into the XhoI and BamHI sites of pcDNA3 [26]. The construction of AE1 SAO by PCR deletion in pcDNA3 is described in [26]. Mutation of the endogenous N-glycosylation site to give AE1 N642D was described in [27]. The AE1 N555 N-glycosylation mutant (with a novel glycosylation site at $Tyr^{555} \rightarrow Asn/Val^{557} \rightarrow Thr$ and the mutation $Asn^{642} \rightarrow Asp$ to eliminate the endogenous glycosylation site) has been described previously [28]. The SAO N642D mutant was created using the QuikChange™ mutagenesis kit using complementary mutagenic primers, with AE1 SAO as the template. SAO N555 was made using the QuikChange™ mutagenesis kit and SAO N642D as template. Kidney isoform constructs were constructed by creating an additional XhoI site 20 bases upstream of Met^{66}, restriction-digesting with XhoI and religating the plasmid [29]. The C-terminal His_6-tagged constructs were made by PCR, inserting codons for six histidine residues immediately after the codon for C-terminal Val^{911}, as described in [29]. Externally HA-tagged or myc-tagged constructs were created by PCR insertion after position 557 in the amino acid sequence as described in [30].

Retroviral expression plasmids pFBneo-kAE1 · HA557, pFBneo-kAE1 SAO · HA557 and pFBneo-kAE1 · myc557 were constructed by inserting the cDNA encoding the entire kAE1 with an external HA-tag or myc-tag at position 557 into the XhoI site of the retroviral expression vector pFBneo.

Sequencing of the constructs was carried out by the ACGT Corp.

Transient transfection and expression of AE1 mutants in HEK-293 cells

HEK-293 cells were grown in DMEM supplemented with 10 % (v/v) calf serum, 0.5 % penicillin and 0.5 % streptomycin under 5 % CO_2 at 37 °C, as described previously [26]. Cells were transfected by the calcium phosphate method [31] with 1 μg of plasmid DNA per well of a six-well plate.

Viral infection and expression of AE1 mutants in MDCK cells

MDCK cells were grown in DMEM/F-12 supplemented with 10 % (v/v) calf serum, 0.5 % penicillin and 0.5 % streptomycin under 5 % CO_2 at 37 °C. For stable transfection, HEK-293 cells were co-transfected with 4 μg each of the three retroviral plasmids, pVpack-GP, pVpack-VSVG, and pFBNeo-kAE1 · HA557, pFBNeo-kAE1 SAO · HA557 and/or pFBNeo-kAE1 · myc557, using FuGene 6 Transfection reagent. Supernatant was collected 24–36 h later, filtered using 0.45 μm filters and added to 30 % confluent MDCK cells in the presence of 8 μg/ml polybrene. The infected cells were selected with 1 mg/ml geneticin G418. Polarized MDCK cells were obtained by growing confluent cells on semi-permeable Transwell polycarbonate filters for 4–5 days.

Enzymic deglycosylation

Cell extracts were prepared by solubilization in 1 % (v/v) $C_{12}E_8$ [26]. Insoluble material was removed by centrifugation at 14 000 ***g*** for 15 min. Cell extract (50 μl) was incubated at room temperature (22 °C) for 1 h with no treatment, 1000 units of EndoH or 500 units of PNGase F. After deglycosylation, 1 vol. of 2× Laemmli sample buffer with 4 % (w/v) SDS was added.

SDS/PAGE and immunoblotting

Proteins were resolved by SDS/PAGE (8 % gels) [32] and transferred on to a nitrocellulose membrane [33]. AE1 was detected with a rabbit polyclonal anti-AE1 Ct (C-terminal) antibody directed against a synthetic peptide corresponding to the C-terminal 16 residues of human AE1 [26]; HA-tagged constructs were detected using mouse monoclonal anti-HA antibody. Band intensities were determined using the ImageJ software, ensuring that all exposures were within the linear range. Expression levels were normalized using the band intensity of GAPDH (detected with mouse anti-GAPDH) in each lane. Statistical significance was analysed using Student's t test ($P < 0.05$).

Pulse–chase experiments

HEK–293 cells (transiently transfected and grown at 37 °C for 24 h) or stably transfected MDCK cells were labelled with 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine in methionine- and cysteine-free DMEM for 30 min. The radioactive medium was removed, and the cells were then chased for up to 24 h with DMEM with 10 % (v/v) calf serum and 0.5 % penicillin and streptomycin. Cells were harvested at intervals after washing with PBS by solubilization in 1× RIPA buffer (1 % deoxycholate, 1 % Triton X-100, 0.1 % SDS, 0.15 M NaCl, 10 mM Tris/HCl, pH 7.5, and 1 mM EDTA). DNA was removed by filtering using a dropping bottle with a 20–30 μm filter unit. AE1 was immunoprecipitated with 3 μl of anti-AE1 Ct antibody followed by 30 μl of Protein G–Sepharose. Proteins were eluted with 50 μl of 2× SDS sample buffer at room temperature for 10 min. Proteins were resolved by SDS/PAGE (8 % gels) and detected by autoradiography. Band intensities were determined by NIH Imager or ImageJ software.

Cell-surface biotinylation

The procedure for biotinylation has been described previously [28]. Briefly, cells were treated twice with 1 ml of 0.8 mM NHS-SS-biotin for 15 min at room temperature. Biotinylated proteins in cell extracts were captured using Streptavidin beads and eluted with SDS sample buffer. The presence of AE1 in the total, unbound and bound fractions was detected by immunoblotting with anti-AE1 Ct antibody.

Histidine-tag co-purification

HEK-293 cells transiently transfected with His_6-tagged constructs (with co-transfection of untagged constructs) were harvested with lysis buffer (1 % $C_{12}E_8$ and 5 mM imidazole with protease inhibitors in PBS). Cell lysates were centrifuged at 14 000 ***g*** for 15 min at 4 °C to remove insoluble material. The supernatants were applied to 50 μl of 50 % slurry of Ni-NTA–agarose in binding buffer (0.1 % $C_{12}E_8$ and 5 mM imidazole with protease inhibitors) and incubated for 1 h at 4 °C. Ni-NTA beads were washed with 0.5 ml of wash buffer (0.1 % $C_{12}E_8$ and 20 mM imidazole with protease inhibitors) three times. Bound proteins were eluted with 0.1 % $C_{12}E_8$ and 500 mM imidazole and solubilized in 2× SDS sample buffer.

For [^{35}S]methionine labelling experiments with co-transfected His_6-tagged erythroid and untagged kidney constructs, transfected HEK-293 cells were radiolabelled for 30 min, then immediately washed with PBS, lysed, and subjected to the purification procedure above. After elution from Ni-NTA, samples were desalted using protein desalting spin columns following the

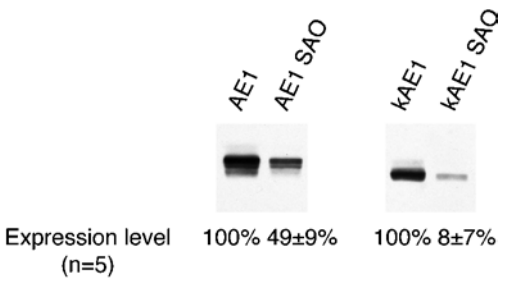

Figure 2 Expression of AE1 and SAO proteins in HEK-293 cells

Cell extracts prepared from HEK-293 cells transiently transfected with AE1 or kAE1 (left-hand panel), or AE1 SAO or kAE1 SAO (right-hand panel) were analysed by SDS/PAGE (8% gels) and immunoblotted with anti-AE1 Ct antibody. Band intensities were measured, and the relative expression levels of AE1 SAO and kAE1 SAO were calculated with AE1 and kAE1 set at 100% respectively. Average expression levels from five experiments are shown beneath each lane ±S.D.

manufacturer's instructions. Immunoprecipitation was then carried out with anti-AE1 Ct antibody and Protein G–Sepharose as described above. Samples were analysed by SDS/PAGE (8% gels) and autoradiography.

Immunofluorescence

MDCK cells (infected with pFBneo-kAE1 · HA557 or pFBneo-kAE1 SAO · HA557, or co-infected with pFBneo-kAE1 · myc557 and pFBneo-kAE1 SAO · HA557) were grown on glass coverslips, or on semi-permeable Transwell polycarbonate filters for 4–5 days after confluence. Cells were fixed with 3.8% (w/v) formaldehyde for 15 min, then washed once with 100 mM glycine, permeabilized with 0.2% (v/v) Triton X-100 for 15 min and then blocked for 30 min with 0.2% (w/v) BSA. Then, 1:1000 diluted mouse anti-HA antibody, 1:250 diluted rabbit anti-myc antibody or 1:500 diluted rabbit anti-AE1 Ct antibody, and 1:200 diluted rabbit polyclonal anti-CNX antibody or 1:1000 diluted mouse monoclonal anti-E-cadherin antibody were added in 0.2% BSA for 30 min. After several washes, samples were incubated with 1:1000 dilution of Alexa Fluor® 488-conjugated anti-mouse antibody or Cy3-conjugated anti-rabbit antibody for 30 min. A Zeiss laser confocal microscope LSM 510 or a Zeiss deconvolution fluorescence microscope was used to observe the samples.

RESULTS

SAO deletion caused reduced AE1 expression in transfected HEK-293 cells

The steady-state expression levels of normal and SAO proteins were examined in transiently transfected HEK-293 cells. Figure 2 shows a representative immunoblot of whole-cell extract, with average expression levels ($n = 5$; ±S.D.) shown beneath each lane normalized to GAPDH levels. The expression levels of AE1 and kAE1 were set at 100%. Both AE1 SAO and kAE1 SAO consistently had a significantly lower expression level than their respective normal counterparts in independent transfection experiments. The reduction in expression level is especially great for kAE1 SAO, averaging less than 10% of kAE1. The lower-molecular-mass band sometimes seen in both AE1 and AE1 SAO samples may be a proteolytic fragment.

SAO mutants had decreased stability in transfected HEK-293 cells

Because of the lower expression level of SAO proteins in transiently transfected HEK-293 cells, we examined their stability

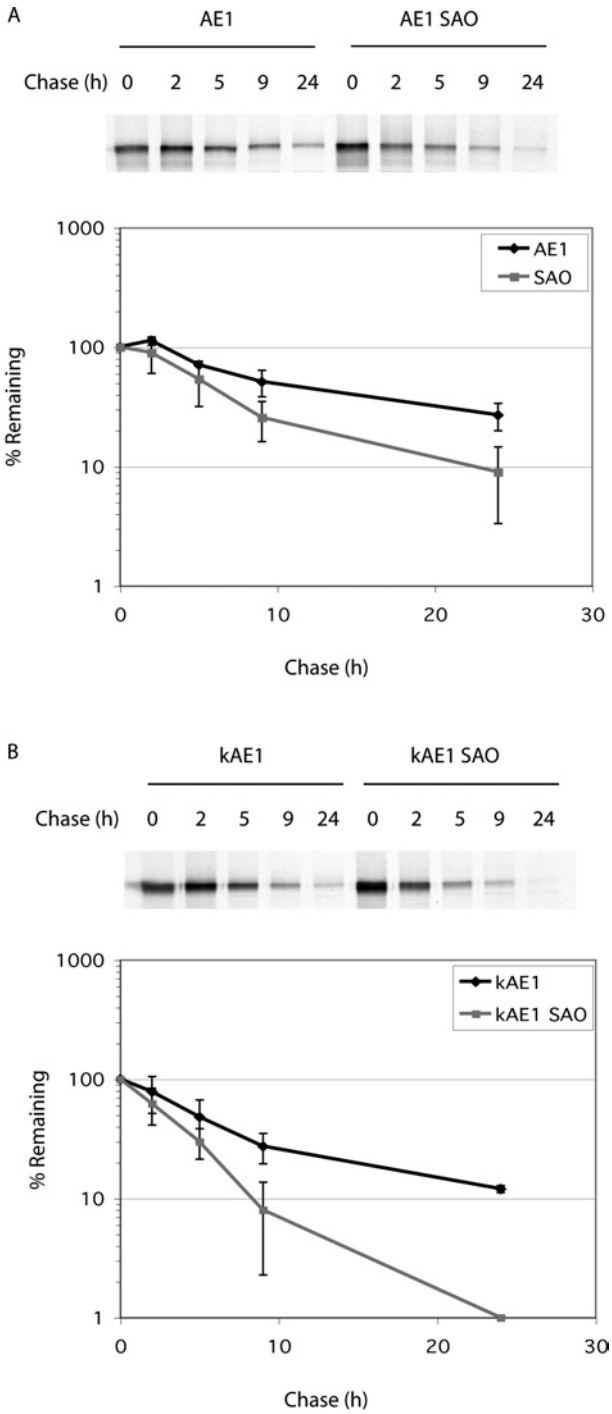

Figure 3 Stability of AE1 and SAO proteins in HEK-293 cells

Transiently transfected HEK-293 cells were labelled with [^{35}S]methionine for 30 min and chased for up to 24 h. At each time point, cells were lysed, and AE1 or AE1 SAO (**A**) or kAE1 or kAE1 SAO (**B**) was immunoprecipitated with anti-AE1 Ct antibody. Samples were resolved by SDS/PAGE (8% gels) and visualized by autoradiography. Top panels are representative autoradiographs. Bottom panels are quantification from three experiments.

by pulse–chase experiments. At 1 day post-transfection, HEK-293 cells were labelled with [^{35}S]methionine for 30 min, then chased for up to 24 h. Autoradiographs and quantification of three experiments are shown in Figures 3(A) (erythroid isoforms) and 3(B) (kidney isoforms). In these experiments, AE1 had a half-life of more than 10 h, in agreement with previous observations

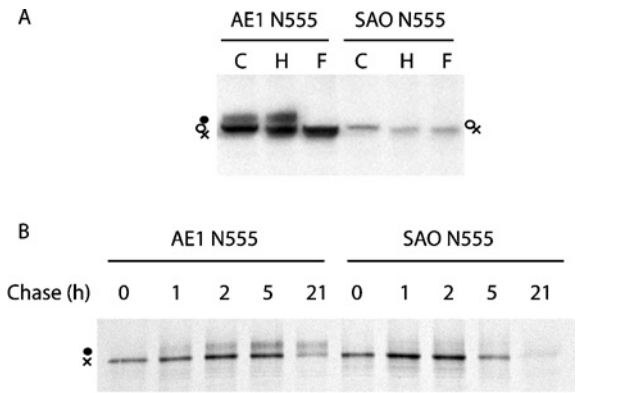

Figure 4 Processing of N555 N-glycan in HEK-293 cells

(**A**) Immunoblot of cell lysates from transiently transfected HEK-293 cells expressing AE1 N555 or SAO N555, detected with anti-AE1 Ct antibody. ● and ○ mark the positions of complex N-glycosylated protein and high-mannose-glycosylated protein respectively. × indicates the position of deglycosylated protein. C, untreated control; H, EndoH-treated sample; F, PNGase F-treated sample. (**B**) Autoradiograph of pulse–chase experiment showing N-glycan processing of AE1 N555 and SAO N555 in HEK-293 cells. Transiently transfected cells were labelled with [^{35}S]methionine and chased for 21 h. AE1 proteins were immunoprecipitated from cell lysates with anti-AE1 Ct antibody and solubilized in sample buffer. Samples were treated with EndoH for better resolution of bands. SDS/PAGE (8 % gels) and autoradiography of the samples were carried out. Lower bands (×) correspond to deglycosylated high-mannose proteins; upper bands (●) represent complex glycosylated proteins.

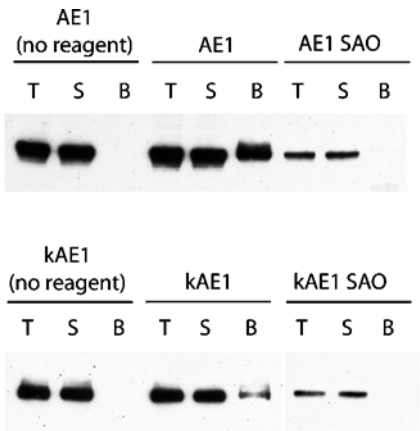

Figure 5 Cell-surface biotinylation of AE1 and SAO proteins in HEK-293 cells

Cell-surface biotinylation with the membrane-impermeant reagent NHS-SS-biotin was carried out on HEK-293 cells transiently transfected with AE1 constructs. Negative control experiments (AE1, no reagent and kAE1, no reagent) were carried out in the absence of NHS-SS-biotin. Total cell lysate (T), supernatant not bound by streptavidin beads (S) and the fraction bound by streptavidin beads (B) were analysed by SDS/PAGE (8 % gels) and immunoblotting against AE1 Ct.

[34,35]. The half-life of AE1 SAO was shorter than AE1, at approx. 5 h (Figure 3A). Interestingly, the half-lives of the kidney isoforms (Figure 3B) were shorter compared with those of the erythroid isoforms. kAE1 had a half-life of 5 h, and that of kAE1 SAO was shorter at approx. 3 h. At 24 h, more than 10 % of kAE1 remained, but no signal for kAE1 SAO was detected, an observation consistent in all experiments (Figure 3B, and results not shown). The presence of the proteasome inhibitor MG262 had some protective effect on the degradation of both AE1 and AE1 SAO (results not shown), suggesting that the proteasome was involved in degradation of the proteins in HEK-293 cells.

SAO mutants were not trafficked to transfected HEK-293 cell surfaces

To determine whether the SAO deletion affects trafficking of AE1, AE1 N555 and SAO N555 constructs were expressed in transiently transfected HEK-293 cells. N-glycosylation is a co-translational process and, as a protein traffics through the ER (endoplasmic reticulum) and Golgi, the N-glycan is usually modified by processing enzymes. The initial high-mannose structure found in the ER and *cis*-Golgi is converted into a complex structure in the medial Golgi. Normal AE1, when expressed in HEK-293 cells, traffics to the plasma membrane without processing of the N-glycan at Asn^{642} [28]. AE1 N555, with the endogenous N-glycosylation site mutated (N642D) and a novel site created in extracellular loop 3 (Y555N, V557T), could acquire a complex-type N-glycan when expressed in HEK-293 cells [28]. The complex N-glycosylated protein was expressed at the cell surface, at approx. 30 % of the total AE1 N555 at steady state [28]. N555 constructs have been useful for monitoring the trafficking of AE1 in HEK-293 cells [29,30,34–37]. Expression of AE1 N555 in HEK-293 cells resulted in two bands when analysed by SDS/PAGE (8 % gels) and immunoblotting (Figure 4A). The lower band was sensitive to EndoH treatment and corresponds to high-mannose N-glycosylated AE1 N555. The upper band was resistant to EndoH and represents complex N-glycosylated AE1 N555. Both bands were sensitive to PNGase F, which digests both high-mannose and complex N-glycans. SAO N555 presented as one band on the immunoblot (Figure 4A). Again, decreased expression level of the SAO construct compared with that of the AE1 construct was evident. The SAO N555 band ran at a similar position to the lower band of AE1 N555, and was sensitive to digestion by EndoH, indicating that it bore a high-mannose N-glycan. This suggests that AE1 SAO had not reached the medial Golgi and had probably not exited the ER in transfected HEK-293 cells.

[^{35}S]Methionine pulse–chase in HEK-293 cells transfected with either AE1 N555 or SAO N555 showed that, while AE1 N555 clearly acquired complex N-glycan at 2-h chase which persisted for at least 21 h, only a trace upper band was seen in SAO N555 samples throughout the chase period (Figure 4B). The autoradiograph (Figure 4B) also showed reduced stability of SAO N555 compared with that of AE1 N555, consistent with the observation seen with AE1 SAO and AE1 (Figure 3A). The N555 results suggest that the trafficking of AE1 SAO in HEK-293 cells is defective.

To examine whether SAO proteins were indeed not trafficked to the plasma membrane, cell-surface biotinylation was carried out with transiently transfected HEK-293 cells. Whereas AE1 was present in the biotinylated fraction, AE1 SAO was absent (Figure 5). kAE1 could also be biotinylated at the cell surface of transfected HEK-293 cells, but not kAE1 SAO (Figure 5). The slight mobility shift sometimes seen in the biotinylated fraction (for example, Figure 5, AE1 lane B) was not due to N-glycan processing [34], but may be a result of modification by the biotinylating reagent. These results showed that the SAO deletion impairs cell-surface expression of AE1 proteins in transfected HEK-293 cells.

Heterodimerization of wild-type and SAO proteins in transfected cells

The trafficking of AE1 SAO in transfected HEK-293 cells was quite different from that in SAO erythrocyte precursors, where AE1 SAO constitutes approximately half of the AE1 content present in the plasma membrane of the mature erythrocyte [14]. To examine whether the presence of normal AE1 protein could

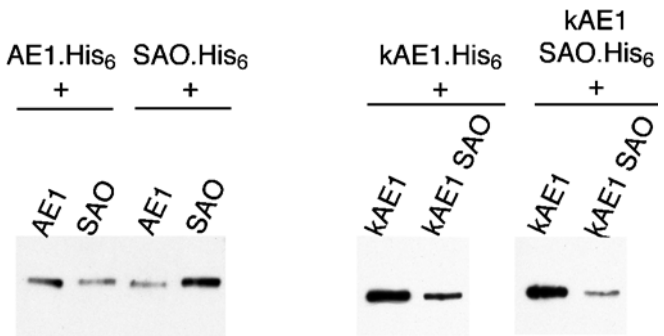

Figure 6 Oligomerization between normal and SAO proteins in HEK-293 cells

HEK-293 cells transiently co-transfected with a His_6-tagged construct and an untagged construct were lysed, and His_6-tagged oligomers were purified by Ni-affinity resin. Purified proteins were eluted and analysed by SDS/PAGE (8 % gels) and immunoblotting. Shown are immunoblots probed with anti-AE1 Ct antibody, which detected the C-terminus of untagged subunits co-purified with the His_6-tagged subunits.

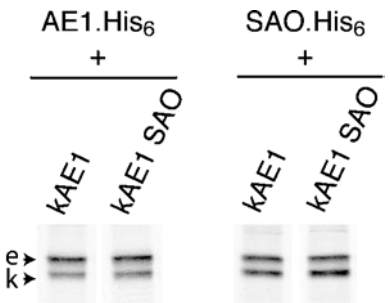

Figure 7 Early association of hetero-oligomer subunits during biosynthesis

HEK-293 cells transiently co-transfected with a His_6-tagged erythroid construct and an untagged kidney construct were labelled with [^{35}S]methionine for 30 min. The cells were then lysed immediately, and His_6-tagged oligomers were purified by Ni-affinity. Eluted proteins were subjected to immunoprecipitation by anti-AE1 Ct antibody, which pulled-down oligomers with at least one untagged subunit. The samples were then analysed by SDS/PAGE (8 % gels) and autoradiography. Shown are autoradiograph results from the zero time chase. e, His_6-tagged AE1; k, untagged kAE1.

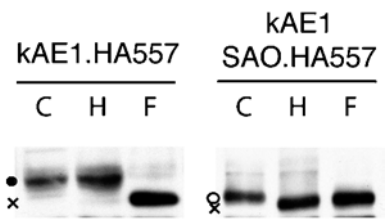

Figure 8 N-glycan processing of kAE1 and kAE1 SAO in MDCK cells

Immunoblots of cell lysates from stably transfected MDCK cells. HA-tagged kAE1 and kAE1 SAO were detected with anti-HA antibody. ●, ○ and × indicate positions of complex-glycosylated kAE1, high-mannose kAE1 and deglycosylated kAE1 respectively. C, untreated control; H, EndoH-treated; F, PNGase-treated.

rescue the trafficking of SAO protein to the cell surface, we first established whether heterodimers could form between normal and SAO proteins in HEK-293 cells by using a co-purification procedure. C-terminally His_6-tagged constructs and untagged constructs were co-transfected into HEK-293 cells. Cell lysates were prepared and were added to Ni-NTA resin for incubation. Only AE1 hetero- or homo-dimers containing one or two tagged subunits respectively would be purified. Homodimers containing only untagged proteins did not bind to Ni-NTA ([29], and results not shown). Proteins bound to Ni-NTA were eluted and analysed by SDS/PAGE (8 % gels) and immunoblotting. The presence of the His_6-tag at the C-terminus prevents the anti-AE1 Ct antibody from recognizing any His_6-tagged proteins [29]. Therefore only the untagged subunit(s) from heterodimers could be detected on the immunoblot. Results of co-purification were similar for erythroid and kidney isoforms and are shown in Figure 6. Homodimers of AE1 and also AE1 SAO could form in transfected HEK-293 cells; homodimers of AE1 SAO were previously inferred, but were not directly observed [18]. Heterodimers of AE1 and AE1 SAO could also form, as seen in both AE1–His_6 + SAO and SAO–His_6 + AE1 combinations. The same homo- and heterodimer combinations were also possible for the kidney isoform expressed in HEK-293 cells. As a control, separate cell lysates containing a tagged protein or an untagged protein were mixed before incubation with Ni-NTA. No untagged protein was co-purified in this case (results not shown), demonstrating that the purification procedure was specific for oligomers formed during biosynthesis.

To determine whether dimerization occurs early in biosynthesis, [^{35}S]methionine-labelling of HEK-293 cells co-transfected with His_6-tagged erythroid construct and untagged kidney construct was carried out. Different isoforms (AE1 and kAE1) were co-expressed to distinguish the tagged and untagged proteins by mobility difference. After a 30-min labelling period, cells were lysed immediately, and dimers containing at least one His_6-tagged subunit were purified by Ni-affinity. Proteins eluted were incubated with anti-AE1 Ct antibody, which immunoprecipitated dimers with at least one untagged subunit. This procedure thus allowed the purification of only heterodimers, but not His_6-tagged or untagged homodimers. After a 30-min labelling period, co-purification of His_6-tagged and untagged constructs was possible. Since AE1 exits the ER with a half-time of approx. 4 h [28], the results indicate that dimers formed in the early stages in biosynthesis in HEK-293 cells are probably in the ER (Figure 7). Pulse–chase samples taken at subsequent time points showed no observable difference in the stabilities of kAE1 SAO compared with kAE1 proteins associated with AE1–His_6 (results not shown), suggesting that heterodimerization with wild-type AE1 may play a role in stabilizing the mutant protein in HEK-293 cells.

Trafficking of kAE1 and kAE1 SAO in MDCK cells

Trafficking of kAE1 and kAE1 SAO were compared in MDCK cells, which can be polarized similar to α-intercalated cells in the kidney. MDCK cells do not express endogenous kAE1, as shown by the absence of immunoreactive bands in immunoblots of untransfected MDCK cell lysates blotted with antibodies directed against the N-terminus or C-terminus of kAE1 (results not shown). KAE1 · HA557 and kAE1 SAO · HA557, constructs with an external HA tag in extracellular loop 3 after Val^{557}, were expressed in MDCK cells by viral infection. It was previously shown that the HA557 tag does not impair trafficking of AE1 to the cell surface [30]. Cell lysates were obtained and analysed by SDS/PAGE (8 % gels) and immunoblotting using an anti-HA antibody. KAE1 · HA557 presented as one major band that was resistant to EndoH treatment, indicating the protein existed predominantly as complex N-glycosylated species in infected MDCK cells (Figure 8). KAE1 SAO · HA557, on the other hand, ran as a single band with a lower molecular mass (Figure 8). With EndoH treatment, the band shifted to an even lower molecular mass (Figure 8), consistent with the presence of high-mannose type N-glycan on kAE1 SAO · HA557 in infected MDCK cells. These results suggest that kAE1 SAO is retained in the ER of MDCK cells.

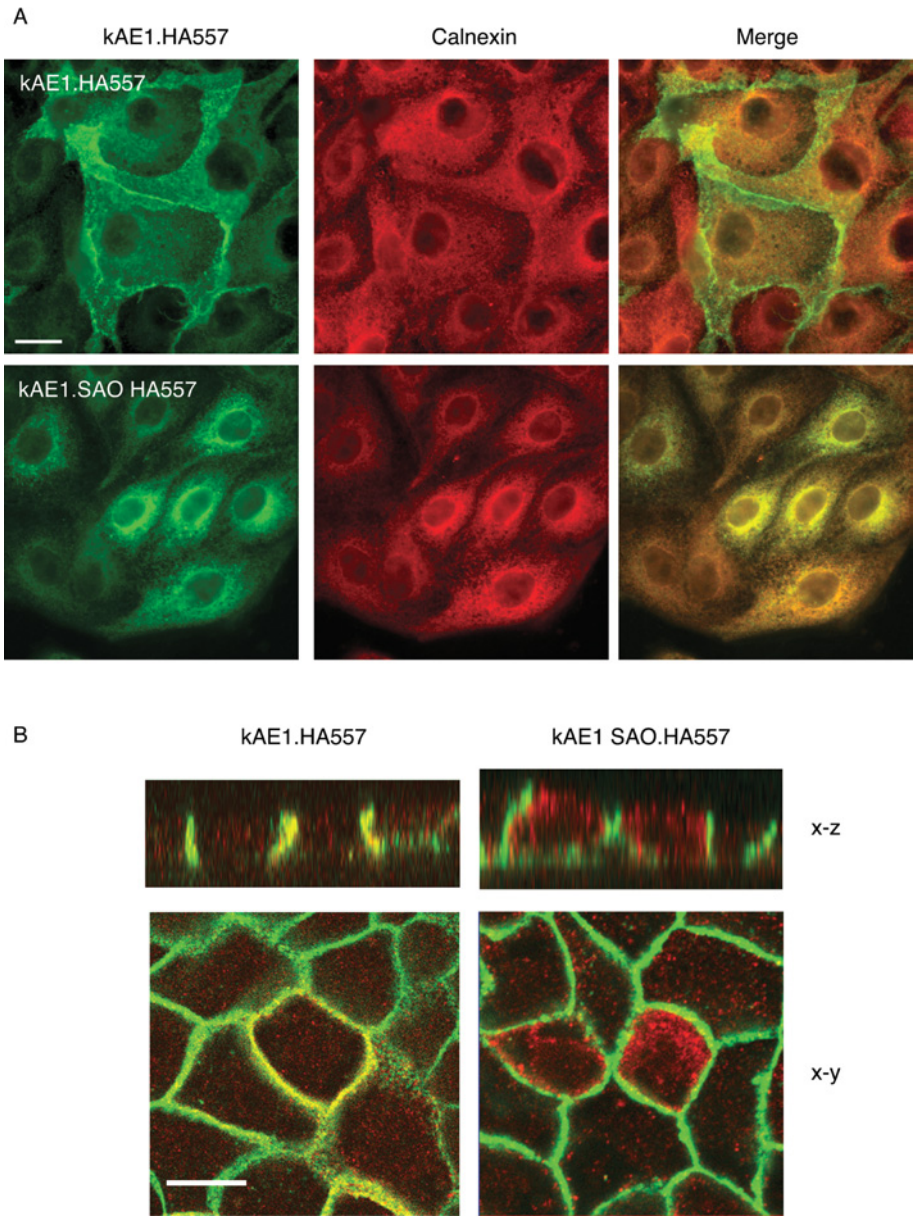

Figure 9 Immunolocalization of kAE1 and kAE1 SAO in MDCK cells

(**A**) Immunofluorescence images of stably transfected unpolarized MDCK cells. Cells grown on coverslips were permeabilized and incubated with mouse anti-HA and rabbit anti-CNX, then with fluorescent secondary antibodies. Red colour shows localization of CNX in the cells, and green represents HA-tagged kAE1 or kAE1 SAO. Scale bar, 10 μm. (**B**) Immunofluorescence images of stably transfected polarized MDCK cells. Cells grown on filters were permeabilized and incubated with rabbit anti-AE1 Ct and mouse anti-E-cadherin antibodies, then with fluorescent secondary antibodies. Green shows the localization of E-cadherin, and red shows the distribution of HA-tagged kAE1 or kAE1 SAO. x-z, cross-section showing side view of cells; x-y, cross-section through the middle of the cell.

In pulse–chase experiments in MDCK cells (results not shown), complex glycosylated kAE1 · HA557 could be detected at 2-h chase, and was present for the duration of the 24-h chase. Complex glycosylated kAE1 SAO · HA557 could also be observed at the 2-h time point, but it was rapidly degraded and was no longer detected at 6-h or 24-h chase. The lower stability of kAE1 SAO compared with wild-type kAE1 in MDCK cells is consistent with results in HEK-293 cells (Figure 3).

Immunofluorescence localization in non-polarized MDCK cells showed that kAE1 · HA557 (Figure 9A, green) expressed predominantly at the cell surface and was not co-localized with the ER marker CNX (Figure 9A, red), consistent with observations in HEK-293 and LLC-PK$_1$ cells [30,37,38] and previous observations of kAE1 expressed in MDCK cells [39,40]. In contrast, kAE1 SAO · HA557 (Figure 9A, green) was intracellular and co-localized with CNX (Figure 9A, red), consistent with the N-glycosylation results. Immunofluorescence assays were also performed on infected MDCK cells grown on filters to form polarized monolayers. KAE1 · HA557 (Figure 9B, red) co-localized with the basolateral surface marker E-cadherin (Figure 9B, green), whereas kAE1 SAO · HA557 (Figure 9B, red) was mainly intracellular and was not detected at the basolateral surface. Results from N-glycan processing and immunofluorescence experiments in MDCK cells show that kAE1 SAO is defective in trafficking from the ER to the cell surface, similar to the observations in HEK-293 cells.

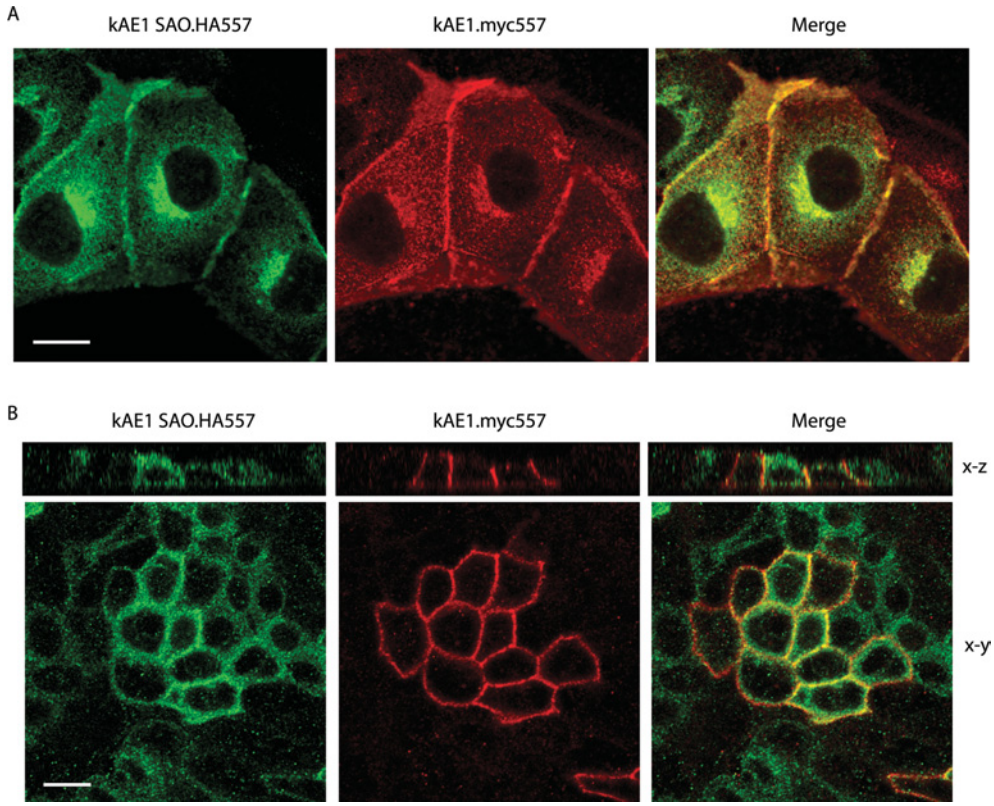

Figure 10 Co-expression of kAE1 and kAE1 SAO in MDCK cells

Immunofluorescence image of MDCK cells stably co-transfected with kAE1 · myc557 and kAE1 SAO · HA557. Unpolarized cells (**A**) were grown on a coverslip and polarized cells (**B**) were grown on a filter. Cells were permeabilized and labelled with rabbit anti-myc and mouse anti-HA, and stained with secondary antibodies. The localization of kAE1 · myc557 and kAE1 SAO · HA557 are shown in red and green respectively. Scale bar, 10 μm. x-z, cross-section showing side view of cells; x-y, cross-section through the middle of the cell.

To study the effect of co-expression of kAE1 and kAE1 SAO on trafficking of the proteins, MDCK cells were co-infected with kAE1 · myc557 and kAE1 SAO · HA557. Immunofluorescence images were taken for non-polarized (Figure 10A) and polarized (Figure 10B) cells. In non-polarized MDCK cells, kAE1 was found not only at the plasma membrane, but also intracellularly where it co-localized with kAE1 SAO. Some kAE1 SAO also co-localized with kAE1 at the cell surface. In polarized MDCK cells, kAE1 and kAE1 SAO co-localized to the BLM (Figure 10B). In these cells, intracellular staining of kAE1 SAO was also noted. These results suggest that kAE1 SAO can retain kAE1 in the ER, but that a fraction of the heterodimers can traffic to the cell surface, particularly in polarized cells.

DISCUSSION

In the present study, we investigated the effect of the SAO deletion on the biosynthesis of AE1 and kAE1 in a non-erythroid mammalian setting. We also examined the trafficking of kAE1 SAO in polarized MDCK cells to simulate the environment of kidney cells. In contrast with previous observations on SAO erythrocytes [14], *Xenopus* oocytes [21,22] and K562 cells [24], erythroid or kidney SAO proteins expressed in HEK-293 or MDCK cells were unstable and defective in trafficking. Co-expression of kAE1 with kAE1 SAO in polarized MDCK cells, mimicking the heterozygous condition of individuals with SAO, promoted the cell-surface expression of kAE1 SAO.

The *Xenopus* oocyte system is quite different from mammalian expression systems. Experiments in oocytes are typically performed at 30 °C, rather than at 37 °C. For CFTR (cystic fibrosis transmembrane conductance regulator), it has been shown that trafficking of the mutant CFTR ΔF508 can be rescued by lowering the temperature [41]. The lower incubation temperature may allow AE1 SAO expressed alone or with normal AE1 to traffic to the oocyte surface [21], but the higher, physiological temperature used for HEK-293 or MDCK cells in our experiments revealed altered trafficking of AE1 SAO in mammalian cell lines. While MDCK cells can form polarized monolayers and are more relevant in the study of kidney AE1 isoforms, HEK-293 cells have been used extensively to study AE1 and are a useful expression system for studying erythroid isoforms and for comparing between erythroid and kidney isoforms.

The fact that in the absence of normal AE1, AE1 SAO can express at the cell surface of K562 cells [24], an erythroleukaemia cell line, but not in HEK-293 cells, suggests that erythroid-specific mechanisms may be at work to rescue the trafficking. One possible mechanism is interaction of AE1 SAO with GPA. Anti-GPA antibodies applied to erythrocytes greatly reduced the mobility of AE1 in the plasma membrane [42], indicating that GPA and AE1 associate in the erythrocyte membrane. A possible site of interaction between AE1 (residue 658) and GPA (Arg^{61}) constitutes the Wright (Wr) blood group antigens [43]. In GPA-deficient M^kM^k erythrocytes, AE1 has a larger N-glycan chain than in normal erythrocytes [44], suggesting that GPA also plays a role in the biosynthesis of AE1. Like AE1 [23], co-expression of GPA and AE1 SAO enhances, but is not necessary for, the cell-surface expression of AE1 SAO in oocytes [21]. The site of GPA interaction with AE1 responsible for facilitating AE1 trafficking probably lies outside the GPA dimerization interface

and may be located in the GPA TM region [45]. In SAO erythrocyte progenitors, GPA may interact with AE1 SAO during biosynthesis. This interaction may stabilize the folding of AE1 SAO enough to escape retention by quality control in the ER, thus allowing AE1 SAO to traffic to the cell surface. Therefore, in the hypothetical case of homozygous SAO, which may be embryonic lethal [13], mature erythrocytes may contain inactive AE1 SAO at the plasma membrane.

Using a histidine-tag co-purification technique, we were able to study the dimerization of AE1 and AE1 SAO proteins. The oligomerization of AE1 in the ER had been previously postulated, but not shown. We provided evidence that the association of subunits occurred early during biosynthesis, probably while the proteins were in the ER. Heterodimerization between mature normal and SAO subunits in SAO erythrocytes had been shown previously [18]; in that study, the existence of SAO homo-oligomers in the plasma membrane was deduced, but not demonstrated directly. The present study shows that SAO proteins can form homodimers. Therefore an intact TM1 is not essential for dimer formation and this region of AE1 is probably not involved in dimerization. Without a high-resolution structure of AE1, however, it is unclear precisely how the dimerization interface might be altered by the SAO deletion. Our work shows that heterodimerization with wild-type protein may increase the stability of the mutant protein.

The expression of kAE1 SAO has not been examined previously. Our work shows that kAE1 SAO is unstable and is more rapidly degraded than kAE1 in transfected HEK-293 cells and infected MDCK cells. Also, the mutant protein, when expressed alone, was retained in the ER of HEK-293 cells and MDCK cells. In the case of homozygous SAO, severe dRTA is expected due to lack of functional kAE1 at the BLM of kidney cells. In MDCK cells co-expressing kAE1 SAO and kAE1, kAE1 SAO was able to traffic to the cell surface, presumably by heterodimerization with kAE1 (Figure 10). The kidney does not express GPA [46]. It is not clear whether other mechanisms exist in α-intercalated cells that can correct the stability and trafficking defect of kAE1 SAO.

In conclusion, both erythroid and kidney SAO proteins are less stable than their normal counterparts in a non-erythroid environment. They are not expressed at the surface of transfected HEK-293 or MDCK cells, and kAE1 SAO is retained in the ER in polarized MDCK cells. In a homozygous state, no functional kAE1 would be present in the BLM in α-intercalated cells in the kidney, resulting in severe dRTA. In the heterozygous state, normal kAE1 dimers and heterodimers with kAE1 SAO would be present at the BLM. Studies in SAO erythrocytes, which contained approx. 50 % SAO protein including some in heterodimers with normal AE1, showed that these erythrocytes retained almost half of the anion-transport activity of normal erythrocytes [16]. Thus the normal subunit is active when associated with an inactive SAO subunit. A similar scenario in the kidney of ovalocytic individuals would probably be sufficient for adequate bicarbonate reabsorption. This is consistent with clinical observations of SAO patients who do not exhibit dRTA [12].

We thank Dr Gergely L. Lukacs for his assistance in the MDCK viral expression system. Jing Li is thanked for her work in subcloning of constructs into vectors.

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Received 4 July 2005/9 August 2005; accepted 18 August 2005
Published as BJ Immediate Publication 18 August 2005, doi:10.1042/BJ20051076